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Assay for and Replication of Karshi (Mammalian Tick-Borne Flavivirus Group) Virus in Mice

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Abstract. Little is known about the replication of Karshi virus, a member of the mammalian tick-borne flavivirus group, in its rodent hosts. Therefore, we developed a quantitative real-time RT-PCR assay and measured the amount of viral RNA in selected tissues of infected Swiss Webster mice. Two-day-old mice were highly susceptible, with 100% fatality 9 to 12 days after infection, whereas infection in 9-day-old mice was less virulent, with death occurring only rarely. In nearly all cases, mice inoculated when 2 days old contained similar numbers of viral genome equivalents from blood and liver samples from any given mouse, with titers declining after day 7. In contrast, the amount of viral RNA in the brain began to rise rapidly 4 days after exposure, peaked at about 6 days after virus exposure (titer of $> 10^{13}$ genome equivalents/g), and remained at that level until euthanasia or death. Viral profiles were similar in needle-inoculated or tick-exposed mice.

INTRODUCTION

Karshi virus (genus *Flavivirus*, family *Flaviviridae*) is a member of the mammalian tick-borne flavivirus group (M-TBFV; previously known as the "tick-borne encephalitis virus [TBEV] serocomplex") and its complete genome was recently sequenced and shown to be most closely related to Royal Farm virus.[1–3] Karshi virus is not considered to be highly pathogenic for humans; however, infections in healthy persons may cause a febrile illness.[4] Severe disease, including encephalitis, has been reported in a few individuals, and large outbreaks of febrile illness associated with infection with Karshi virus have been observed in Uzbekistan (S. Khodjaev, personal communication in Turell et al. 2004[5]).

The natural transmission cycle of members of the M-TBFV complex involves ixodid ticks and rodents[6] and recent studies have also shown that argasid ticks in the genus *Ornithodoros* are competent vectors of these viruses.[5,7] However, little is known about the replication of Karshi virus in its rodent hosts. Preliminary studies indicate that infection of 2-day-old white laboratory mice with Karshi virus uniformly results in a fatal infection, with deaths occurring 8–12 days after infection by either tick bite or subcutaneous (SC) needle inoculation.[5] However, 9-day-old mice rarely die if similarly exposed (MJT, unpublished). Although the ability to measure the Karshi virus present in a sample by plaque assay has been reported in the literature (C. Calisher, personal communication in Karabatsos 1985[8]), we have been unable to plaque or detect cytopathic effects of Karshi virus in any of numerous cell lines (MJT, unpublished). In previous experiments, we quantified the amount of Karshi virus present in a sample by suckling mouse SC lethal $dose_{50}$ ($SMLD_{50}$) units, which is laborious, time-consuming, and expensive. Thus, in the present study, we developed a quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay to specifically measure the amount of viral RNA in selected mouse tissues.

Because of the lack of knowledge about the replication and tissue tropisms of Karshi virus in rodents, we infected young mice either by SC needle inoculation or by allowing an infected tick to feed on them, and then determined and quantified the presence of Karshi viral RNA in selected tissues by time after infection.

MATERIALS AND METHODS

Ticks. A laboratory colony, derived from *Ornithodoros tartakovskyi* ticks provided by the National Institute of Allergy and Infectious Diseases, was maintained as described by Durden et al.[9] Members of this colony were previously shown to be highly efficient transmitters of Karshi virus.[5]

Virus preparation and RNA extraction. The UZ-2247 strain of Karshi virus, isolated from *Hyalomma asiaticum asiaticum* ticks captured in the Kashkadarinsk region of Uzbekistan, had been passaged once in Vero cells and once in suckling mice in our laboratory before use in these experiments. Serial dilutions of the viral inoculum were inoculated SC into 2-day-old suckling mice to estimate the number of $SMLD_{50}$ present. The identity of the original virus was confirmed by a Karshi-specific TaqMan PCR assay, as described below, and by direct sequencing of the PCR products.

Total RNA was extracted from homogenized tissues using TRIzol-LS® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The final RNA pellet was resuspended in 50 μL RNase-free water and stored at −70° until used.

Quantitative RT-PCR assay. Real-time RT-PCR was carried out with the LightCycler 2.0 (Roche, Indianapolis, IN) using oligonucleotide primers and a probe selected from conserved regions of the NS5 gene of Karshi virus. The forward primer sequence was: 5′-CCT GAA TGA CAT GGC GAA AGT-3′, and the reverse primer sequence was: 5′-CCG ATG GAT GGC TCC CA-3′. Primers were synthesized using standard phosphoramidite chemistry and were from Invitrogen (Carlsbad, CA). The TaqMan® -MGB probe (5′-6FAM-AGA AAG GAC ATC GGC G-MGBNFQ-3′) was synthesized by Applied Biosystems (Foster City, CA) and contained 6-carboxyfluorescein at the 5′ end. A non-fluorescent quencher and the minor groove binder protein were added to the 3′ end. One-step RT-PCR was performed using the Invitrogen SuperScript™ One-Step RT-PCR with Platinum® *Taq* kit in a final volume of 20 μL. Reactions contained 5 μL

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14. ABSTRACT

Little is known about the replication of Karshi virus, a member of the mammalian tick-borne flavivirus group, in its rodent hosts. Therefore, we developed a novel quantitative real-time RT-PCR assay and measured the amount of viral RNA in selected tissues of infected Swiss Webster mice. Two-day-old mice were highly susceptible, with 100% fatality between days 9 and 12 after infection, while 9-day-old mice were less susceptible with death occurring only rarely. In nearly all cases, mice inoculated when 2-days old contained similar numbers of viral genome equivalents from blood and liver samples from any given mouse, with titers declining after day 7. In contrast, the amount of viral RNA in the brain began to rise rapidly 4 days after exposure, peaked at about 6 days after virus exposure with a titer of >1013 genome equivalents/g, and remained at that level until euthanasia or death. Viral profiles were similar in needle-inoculated or tick-exposed mice

15. SUBJECT TERMS

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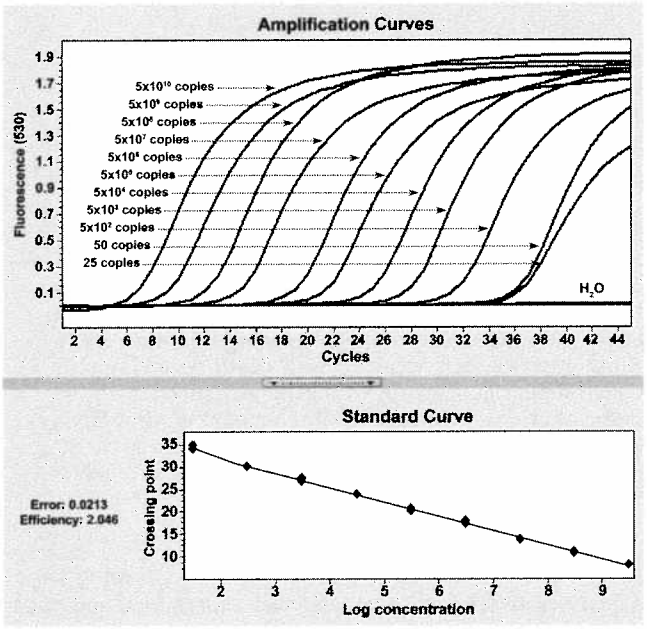

FIGURE 1. Amplification curves (A) and standard curve (B) using synthetic RNA standards assayed with the Karshi virus real-time RT-PCR. The graph shown represents the results from a representative run.

of extracted RNA, 2X reaction mix (containing 0.4 mM of each dNTP), 250 μg/mL of bovine serum albumin, 6.2 mM of $MgSO_4$, 0.5 μM of each primer, 0.1 μM of probe, and 0.25 μL of RT/Platinum® *Taq* Mix. Amplification was carried out with the following cycling conditions: reverse transcription at 50°C for 15 min, initial denaturation at 95°C for 2 min, followed by 45 cycles consisting of denaturation at 95°C for 1 s and annealing/synthesis at 60°C for 20 s.

For quantification of the amount of RNA present, a synthetic 62-nucleotide piece of RNA, encompassing the 57 nucleotides of the entire RT-PCR amplicon (plus two U at the 5′ end and three U at the 3′ end added for stability) was obtained from Dharmacon (Lafayette, CO). The RNA was measured by a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Tenfold serial dilutions of the synthetic RNA were measured and used to generate a standard curve. The LightCycler analysis software version 4.0 was used to generate linear regression curves and accompanying attributes (error and efficiency). Microsoft Excel was used to determine the slope and *r* value of the standard curves.

Experimental design. Two-day-old suckling mice (Swiss Webster strain) were inoculated SC with 0.05 mL of a suspension containing diluent or a 1:300 dilution of a mouse brain suspension in diluent containing 10^5 $SMLD_{50}$ ($10^{6.3}$ $SMLD_{50}$/mL) units of Karshi virus and returned to their dam (one cage with 10 suckling mice inoculated with diluent, and three cages with 10 suckling mice each inoculated with Karshi virus). One mouse per cage was removed at selected times after infection (days 1, 2, 4, 5, 6, 7, 8, 9, 10, and 12), euthanized with CO_2, and 0.1 mL of blood was collected by cardiac puncture. The blood was mixed 1:10 in diluent (Medium 199 with Earle's salts containing 10% heat-inactivated fetal bovine serum and 5 μg of amphotericin B, 50 μg of gentamicin, 100 units of penicillin, and 100 μg of streptomycin per ml and 0.075% $NaHCO_3$). The peritoneal cavity was then opened with sterile scissors and forceps and a portion (~100 mg) of the liver removed. This was triturated in 1 mL of diluent in a glass grinder. We used a 3-mL syringe, with a 16-gauge needle, to remove a portion of the brain (~100 mg), and this was triturated into 1 mL of diluent. A 0.25-mL aliquot of each of these suspensions was removed and added to separate cryovials containing 0.75 mL of TRIzol-LS. The remaining suspension was frozen at −70°C. A similar study was conducted with 9-day-old mice, except that mice were sampled on days 1, 2, 4, 5, 7, 9, 11, 14, 21, and 28 days after virus exposure. On most days, samples were obtained from three virus-inoculated and one diluent-inoculated mouse. Finally, six 2-day-old mice were individually exposed to *O. tartakovskyi* ticks (one tick per mouse for up to 1 hr of exposure) that had been inoculated with Karshi virus > 3 years previously.[5] These individual ticks had each been shown to transmit Karshi virus. Ticks fed on three of these mice, and these mice were returned to their dam. They were euthanized and tested as above 2, 4, or 6 days after tick feeding to compare viral profiles in mice infected by tick bite as compared with those infected by SC needle inoculation. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals.

RESULTS

Characteristics of the quantitative Karshi virus real-time RT-PCR. Tenfold serial dilutions of synthetic RNA were used to produce standard curves for the real-time RT-PCR by

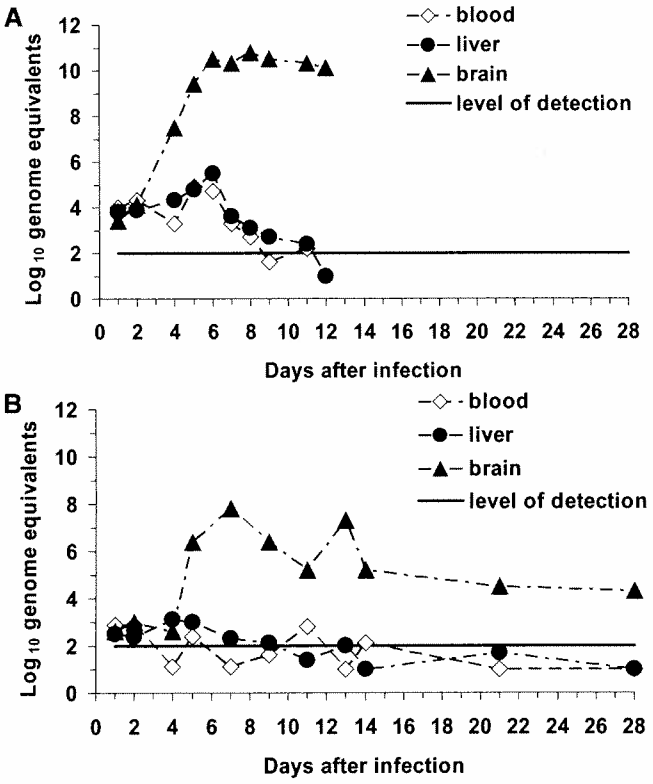

FIGURE 2. Number of genome equivalents per tissue by day after infection for suckling mice infected by subcutaneous inoculation of Karshi virus when 2 days old (**A**) or 9 days old (**B**). For calculation of means, negative samples were assigned a value of 1.0.

TABLE 1
Reproducibility of the Karshi virus RT-PCR*

Genome equivalents	Mean crossing point (range)	SD
5×10^9	9.0 (7.8–10.3)	1.0
5×10^8	11.8 (10.6–13.1)	0.9
5×10^7	14.6 (12.9–16.2)	1.2
5×10^6	17.5 (16.3–18.1)	0.6
5×10^5	20.5 (19.9–21.0)	0.4
5×10^4	23.6 (22.9–24.2)	0.4
5×10^3	26.8 (25.7–27.5)	0.6
5×10^2	29.9 (28.7–30.7)	0.6
5×10^1	33.5 (31.7–35.0)	1.0
2.5×10^1	33.8 (31.7–35.2)	0.9

SD, standard deviation.
* Each sample was tested 12 times (6 separate days by 2 different operators).

plotting the crossing point (cycle threshold) value against copy number (Figure 1). The assay could consistently detect 0.5 attagram of purified RNA, which represents approximately 25 gene copies (genome equivalents). Limit of detection experiments showed linear correlation ($r^2 = 0.99$) with a dynamic range of eight orders of magnitude, representing approximately 50 to 5×10^9 copies (Table 1). As a conservative measurement, we considered 100 genome equivalents to be virus positive in the mouse tissues for this study. As part of this study, we also tested liver, brain, and blood from 17 negative control mice. These tissues contained a mean (95% CI) of 3.6 (1.4–8.6), 4.3 (1.4–11.3), and 3.3 (1.1–9.6) genome equivalents, respectively. To examine specificity, we also tested Russian spring summer encephalitis, Central European encephalitis, and Powassan viral RNA, as well as a specimen "spiked" with West Nile virus. All of these specimens were negative by this assay (data not shown). Additionally, to examine the effect of the presence of various tissues on the detection process, we spiked diluent as well as various tissue samples (liver, brain, and blood) from uninfected mice with several dilutions of our 62-nucleotide synthetic RNA and assayed them. We did not observe a difference in crossing points at any of these dilutions for any of the tissues or between the tissues and diluent.

Replication of Karshi virus in 2-day-old mice inoculated SC. Karshi viral RNA was detected in all mice inoculated SC with Karshi virus. Based on a serial titration in 2-day-old mice and the real-time RT-PCR, we estimated that each $SMLD_{50}$ contained about 80 genome equivalents. In mice that were inoculated with Karshi virus when they were 2 days old, viremias could be detected for nearly 8 days, but titers declined beginning on day 7 (Table 2; Figure 2). In nearly all cases, the numbers of viral gene equivalents detected were similar for blood and liver samples from a given mouse. In contrast, the amount of viral RNA in the brain began to rise rapidly 4 days after virus exposure, peaked about 6 days after virus exposure with a titer of $> 10^{10}$ gene equivalents/mL ($> 10^{13}$ gene equivalents/g of brain tissue), and remained at that level until death (Table 2). Beginning on day 8 after infection, some of the mice developed hind leg paralysis and these mice were selected for testing on that day. None of the mice appeared healthy as of day 11, and all were dead or moribund by day 12.

Replication of Karshi virus in 9-day-old mice inoculated SC. In comparison to mice inoculated when they were 2 days old, mice inoculated with Karshi virus when they were 9 days old developed lower titers of Karshi viral RNA. Viremias were lower and were usually undetectable by 4 days after infection (Table 2; Figure 2). Viral RNA was detected in the liver samples from nearly all mice for 5 days, from about half of mice sampled 7–9 days after infection, and from only 1 of 14 mice sampled from days 11–28. (That one mouse, tested on day 14, contained 10^4 genome equivalents/mL.) Viral RNA was detected from brain samples from all but one of the 32 mice tested. However, beginning on day 4 after infection, titers were about 1,000- to 100,000-fold lower than those from 2-day-old mice sampled on the same day after infection (Table 2).

Replication of Karshi virus in 2-day-old mice fed on by an infectious tick. Karshi viral RNA was detected in three of three mice fed on by an infectious tick. Viral titers recovered from blood, liver, or brain from these mice sampled 2, 4, or 6 days after tick feeding were similar to those obtained from mice tested after a similar interval after being inoculated SC with Karshi virus (data not shown).

DISCUSSION

This is the first report of viral profiles in selected tissues by day after infection in a rodent model for Karshi virus. Use of

TABLE 2
Replication of Karshi virus in selected tissues of mice by day after SC inoculation with 10^5 $SMLD_{50}$ of virus

Days after inoculation	Age at inoculation: 2 days			9 days		
	Blood	Liver	Brain	Blood	Liver	Brain
1	4.0 (0.8)*	3.8 (0.1)	3.4 (0.5)	2.9 (0.5)	2.5 (0.4)	2.6 (0.3)
2	4.2 (0.3)	3.9 (0.2)	4.1 (0.5)	2.9 (0.5)	2.5 (0.3)	3.0 (0.6)
4	3.3 (0.1)	4.3 (0.2)	7.5 (0.5)	Neg	3.5 (0.4)	2.6 (1.1)
5	4.9 (0.8)	4.8 (0.4)	9.4 (0.3)	2.4 (0.5)	3.0 (0.7)	6.4 (0.8)
6	4.7 (0.4)	5.5 (0.4)	10.5 (0.2)	N.T.	N.T.	N.T.
7	3.3 (0.5)	3.6 (0.2)	10.3 (0.2)	Neg	2.3 (0.7)	7.8 (1.1)
8	2.7 (0.7)	3.1 (0.8)	10.8 (0.2)	N.T.	N.T.	N.T.
9	Neg	2.7 (0.5)	10.5 (0.3)	Neg	2.1 (1.1)	6.4 (0.3)
11	2.2 (0.7)	2.4†	10.3 (0.1)	2.8 (1.0)	Neg	5.2 (0.2)
12	Neg	Neg	10.1 (0.1)	Neg†	Neg†	7.3†
13	N.T.	N.T.	N.T.	Neg	Neg	5.2 (1.2)
14	N.T.	N.T.	N.T.	2.1 (1.1)	Neg	5.2 (1.2)
21	N.T.	N.T.	N.T.	Neg	Neg	4.5 (0.6)
28	N.T.	N.T.	N.T.	Neg	Neg	4.3 (0.3)

Neg, negative (mean logarithm_{10} genome equivalents ≤ 2.0); N.T., not tested.
* Mean logarithm_{10} genome equivalents per mL (S.E.). Three mice were tested at each point except as indicated.
† Only one mouse tested.

RT-PCR technology enabled us to demonstrate that viral RNA persisted in the blood for about a week and that virus crossed the blood–brain barrier and initiated an infection in the brain about 3 or 4 days after infection. Extremely high titers of genome equivalents, often exceeding 10^{13} copies per g of brain tissue, were detected in all mice inoculated with Karshi virus when they were 2 days old when assayed ≥ 6 days after infection.

Karshi virus is a member of the M-TBFV group, previously referred to as the TBEV serocomplex due to its close relationship with this group of viruses. Although the complete genome of Karshi virus was recently determined[3] and limited studies on its vector competence have been published,[5] little is known about its pathogenicity for its mammalian host. Our studies showed that viremias may persist in a rodent for 4–8 days, depending on the age of the rodent at the time of infection, and previous studies indicate that these viremias are sufficient to infect argasid ticks, including *Ornithodoros tartakovskyi*, a species found where Karshi virus is enzootic.[5]

We developed a quantitative real-time RT-PCR assay that was able to detect as few as 50 genome equivalents of Karshi virus and was able to measure the number of genome equivalents of Karshi virus RNA in selected mouse tissues by time after infection. The high sensitivity of the assay reliably detected virus in the early stages of the infection and persistence of viral genomes for at least 28 days in the brains of mice that survived infection. The pattern of viral replication in mice was similar to the pattern in mice infected by the bite of an infective tick or by SC inoculation with virus. Few data on the pathogenic nature of Karshi virus exist in the literature. However, encephalitis has been reported in a few individuals (S. Khodjaev, personal communication in Turell et al. 2004[5]). This report of viral profiles in selected tissues by day after infection in a rodent model for Karshi virus supports the assumption of neurotropism by this virus, and suggests that further investigation into the pathogenicity of Karshi virus in humans is warranted.

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